ENVIRONMENTAL RESEARCH 5, 127–134 (1972)

Techniques of Sensitization of Guinea Pigs with Chromium Salts A Comparative Study[1]

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Received June 28, 1971

Three methods of sensitizing guinea pigs to chromium salts are compared. The protocols differed in regard to the concentration of antigen, the route of injection, the methods of reinforcement, the volume injected and simultaneous injection versus splitting of the adjuvant. The split adjuvant method of Maguire, in which the chemical allergen is injected first, resulted in good levels of sensitization in 100% of the animals. This method involved intradermal injections of small amounts of a dilute chromium solution, followed shortly by injection of Freund's complete adjuvant. Though slightly more intense reactions were achieved by the method of Gross *et al.*, the Gross protocol requires three weekly subcutaneous doses of a larger volume, delivered as an emulsion containing Freund's complete adjuvant. A third method involved intramuscular injection, a larger total volume of chromium, and reinforcement doses containing rather concentrated aqueous solutions. It did not achieve comparable success.

INTRODUCTION

In the guinea pig, induction of delayed hypersensitivity to chemical allergen salts has been shown to vary with age, the genetic strain, the species of salt, the concentration of antigen, the dosage schedule, the site of sensitization, and the route of sensitization, as well as with the use of adjuvants. Therefore, the efficacy of one technique of sensitization relative to other techniques is best studied in one strain of guinea pig that is known to consistently develop delayed hypersensitivity. We reported previously a technique for consistent induction in guinea pigs of high levels of sensitization to chromium salts (Gross, Katz, and Samitz, 1968), and we were interested in comparing our experimental model with that of Polak *et al.* (1968), a method which resulted in the quick induction of sensitization to metal compounds, including chromium.

In another study, Maguire and Chase (1967) reported exaggerated delayed-type hypersensitivity to simple chemical allergens in the guinea pig. Their technique involved splitting the adjuvant and the chemical allergen, followed by reinforcement through the epicutaneous application of the chemical allergen. "Supersensitivity" was achieved with three potent chemical sensitizers (picric

[1] This research was supported under Grant EC 00157 Environmental Control Administration, Consumer Protection and Environmental Health Service, Public Health Service, Department of Health, Education, and Welfare.

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acid, picryl chloride and dinitrochlorobenzene). In a subsequent paper (Maguire, 1968), the split adjuvant technique was reported as also successful in producing hyperacute enhancement of delayed hypersensitivity to purified proteins (ovalbumin) in the guinea pig. We thought it advisable also to test this split adjuvant technique using chromium.

METHODS

I. Method of Gross et al. (*1968*)

Fourteen albino guinea pigs weighing 300–500 g were sensitized to hexavalent chromium ($K_2Cr_2O_7$) by three subcutaneous injections in the nape of the neck 1 week apart. The emulsion injected consisted of 0.5 mg of Freund's complete adjuvant (Difco) with 0.5 ml of 3.4×10^{-3} M of $K_2Cr_2O_7$. Ten pigs were challenged at 6 weeks by intradermal injections on clipped skin using 0.1 ml of 4.2×10^{-4} M of $K_2Cr_2O_7$ and 0.1 ml of 8.5×10^{-4} M of $K_2Cr_2O_7$. Reactions were read at 48 hours. Simultaneous testing of four pigs to conform to the schedule for split adjuvant technique was also carried out with concentrations of 4.2×10^{-4} M of $K_2Cr_2O_7$ only. Reactions were read at 48 hours, on an arbitrary scale as follows: 0 = no reaction or a barely perceptible trace of erythema; + = well-defined erythematous patch with no induration; + + = large erythematous patch with induration; + + + = large erythematous plaque with induration and vesiculopustule formation.

II. Method of Polak et al. (*1968*)

Ten albino pigs weighing 300–500 g were injected intramuscularly with 1 mg of $K_2Cr_2O_7$ in 1 ml of Freund's complete adjuvant (Difco), corresponding to 3.4×10^{-3} M of $K_2Cr_2O_7$. In an attempt to reinforce sensitization 2 weeks later, they were injected intradermally with 25 mg of $K_2Cr_2O_7$ in 0.1 ml of 0.05 M of NaCl, corresponding to 0.85 M of $K_2Cr_2O_7$. This was repeated at weekly intervals. Also beginning 2 weeks after the initial sensitization, the animals were painted, on clipped skin, with 0.5% $K_2Cr_2O_7$ in 1% Triton × 100 at weekly intervals, corresponding to 1.7×10^{-2} M of $K_2Cr_2O_7$. Six weeks after the initial sensitization dose, the animals were challenged with intradermal injections, on clipped skin, using 0.1 ml of 4.2×10^{-4} M of $K_2Cr_2O_7$ and 8.5×10^{-4} M of $K_2Cr_2O_7$. Reactions were read at 48 hours.

III. Split Adjuvant Technique (*Maguire, 1967*)

A. *Adjuvant first.* Each of four albino guinea pigs weighing 300–500 g received intradermal injections of 0.3 ml of Freund's complete adjuvant (Difco) at five different sites simultaneously in one flank. Twenty-four hours later, each received 0.3 ml of 3.4×10^{-3} M of $K_2Cr_2O_7$ injected into the wheals produced by the injection of Freund's adjuvant. Two weeks later two of the pigs received topical application of 0.5% $K_2Cr_2O_7$ in 1% Triton × 100, on shaved skin, and this was repeated on shaved as well as tape-stripped skin, the following week. The remaining two pigs received two weekly intradermal injections with 0.1 ml of

4.2×10^{-4} M of $K_2Cr_2O_7$ 2 weeks after the sensitizing dose. Intradermal testing was carried out 4 and 7 weeks after initiation of sensitization.

B. *Antigen first.* Each of five albino guinea pigs weighing 300–500 g received intradermal injections of 0.3 ml of 3.4×10^{-3} M of $K_2Cr_2O_7$ at different sites on one flank. One and one-half hours later each guinea pig received 0.3 ml of Freund's complete adjuvant injected into the wheals of the injection. Two weeks later three of the pigs received a topical application of 0.5% $K_2Cr_2O_7$ in 1% Triton × 100 on shaved skin. This was repeated on shaved skin as well as tape-stripped skin the following week. The remaining two pigs received two weekly intradermal injections of 0.1 ml of 4.2×10^{-4} M of $K_2Cr_2O_7$ 2 weeks after the sensitizing dose. Intradermal testing was carried out at this same concentration 4 and 7 weeks after initiation of sensitization.

RESULTS

I. *Comparison of Methods of Gross* et al. *with Polak* et al.

Method of Gross et al. (*1968*). Challenge testing at 6 weeks showed induction of sensitivity to $K_2Cr_2O_7$ in 100% of the animals. Degrees of sensitization to intradermal testing at 8.5×10^{-4} M of $K_2Cr_2O_7$ showed an average graded reaction

TABLE I

COMPARISON OF INTRADERMAL REACTIONS AT TWO CONCENTRATIONS OF $K_2Cr_2O_7$ IN GUINEA PIGS SENSITIZED BY THE METHODS OF GROSS *et al.* AND BY THE METHODS OF POLAK *et al.*

		8.5×10^{-4} M $K_2Cr_2O_7$	4.2×10^{-4} M $K_2Cr_2O_7$
Method of Gross *et al.* (1)	1.	3+	2+
	2.	3+	3+
	3.	2+	2+
	4.	2+	1+
	5.	2+	2+
	6.	2+	1+
	7.	3+	2+
	8.	2+	2+
	9.	2+	1+
	10.	2+	2+
Method of Polak *et al.* (2)	1.	0	0
	2.	0	0
	3.	0	0
	4.	1+	0
	5.	0	0
	6.	0	0
	7.	1+	0
	8.	0	0
Controls	1.	0	0
	2.	0	0
	3.	0	0
	4.	0	0

of +2.3. The graded reactions at 4.2×10^{-4} M of $K_2Cr_2O_7$ showed an average graded reaction of +1.8.

Method of Polak et al. (*1968*). Challenge testing at 6 weeks showed induction of sensitivity in only 25% of the animals. Degrees of sensitization to intradermal testing at 8.5×10^{-4} M of $K_2Cr_2O_7$ showed an average reaction of +0.25. With concentrations of 4.2×10^{-4} M of $K_2Cr_2O_7$ there were no positives. The results are tabulated in Table I.

II. Comparison of Split Adjuvant Technique (Maguire, 1967) with Method of Gross et al.

The group of guinea pigs that received adjuvant followed 24 hours later by $K_2Cr_2O_7$, failed to become sensitized and were the same as the control animals on subsequent testing.

The group of guinea pigs that received $K_2Cr_2O_7$ first, followed 90 minutes later by the adjuvant, showed 100% induction of sensitivity by the 20th day of testing,

TABLE II

COMPARISON OF INTRADERMAL REACTIONS TO $K_2Cr_2O_7$ (4.2×10^{-4} M) IN GUINEA PIGS SENSITIZED VIA THE METHODS OF THE SPLIT ADJUVANT TECHNIQUE AND THAT OF GROSS *et al.*

Animal	Day 0	Day 14	Day 16 reading	Day 21	Day 23 reading	Day 30	Day 32 reading	Day 49	Day 51
Group #1 Antigen first (split adjuvant)									
1	Ant. 1st	Painting	0	Painting	1+	Inj.	1+	Inj.	1+
2	Ant. 1st	"	0	"	1+	Inj.	1+	Inj.	2+
3	Ant. 1st	"	0	"	0	Inj.	1+	Inj.	1+
4	Ant. 1st	Intrad. Inj.	0	Intrad. Inj.	2+	Inj.	2+	Inj.	2+
5	Ant. 1st	Intrad. Inj.	0	Intrad. Inj.	1+	Inj.	1+	Inj.	1+
Group #2 Adjuvant first (split adjuvant)									
1	Adj. 1st	Painting	0	Painting	0	Inj.	0	Inj.	0
2	Adj. 1st	"	0	"	0	Inj.	0	Inj.	0
3	Adj. 1st	Intrad. Inj.	1+	Inj.	0	Inj.	0	Inj.	0
4	Adj. 1st	Intrad. Inj.	TR	Inj.	0	Inj.	0	Inj.	0
Group #3 (Method of Gross *et al.*)									
	Day 0	Day 7	Day 14						
1	Inj.	Inj.	Inj.			Inj.	2+	Inj.	2+
2	Inj.	Inj.	Inj.			Inj.	2+	Inj.	3+
3	Inj.	Inj.	Inj.			Inj.	1+	Inj.	3+
4	Inj.	Inj.	Inj.			Inj.	1+	Inj.	1+
Control									
1								Inj.	0
2								Inj.	0
3								Inj.	0
4								Inj.	0

with an average graded reaction of +1.2 to 4.2×10^{-4} M of $K_2Cr_2O_7$. By Day 51 the average reaction increased to +1.4.

A group of guinea pigs sensitized simultaneously by the technique of Gross *et al.* were sensitive by Day 30 and showed an average graded reaction to 4.2×10^{-4} M of $K_2Cr_2O_7$ of +1.5, with increase to +2.3 on retesting by Day 51. These differences are tabulated in Table II.

DISCUSSION

The split adjuvant technique has been reported effective with strong chemical allergens such as DNCB, picric acid and picryl chloride. $K_2Cr_2O_7$ was not used by Maguire in his studies. We were interested in the possibility of further enhancement of sensitization to chromate by the alternative method of splitting the adjuvant schedule. The method of split adjuvant that most closely resembled our technique (chemical allergen first, separated by 90 minutes from the adjuvant) produced good though slightly lower levels of response. Biologically it is possible that such a splitting of antigen and adjuvant (by 90 minutes) is inconsequential in the chain of immunologic events that follow. Magnusson and Kligman (1970), using a stronger allergen, *di-t*-butylphenyldisulfide (BPS), found that delaying the injection of Freund's adjuvant for 1 to 4 days after antigen injection, resulted in as effective a rate of sensitization as simultaneous injection, and no superior results were reported.

Hypothetical advantages to a simultaneous injection of Freund's complete adjuvant and some antigens include protection from rapid cellular destruction by a water-in-oil emulsion of antigen, distribution of antigen throughout the body in oil globules, and the induction of permeability changes or cellular damage, which makes the antigen more accessible to the appropriate antibody forming cells (Freund, 1956; Munoz, 1964).

Although Freund originally proposed that the adjuvant and antigen be utilized as an emulsion, the success of this modification of the split adjuvant technique clarifies the fact that an emulsion is not necessary. Although the same site was injected by the split adjuvant technique, Magnusson and Kligman compared the effectiveness of a variety of allergen-adjuvant mixtures injected at separate sites about 5–10 mm apart in the nuchal area. Results varied with the different antigen systems, although it is of interest that in the case of several potent allergens, separate injections resulted in higher sensitization responses. This was interpreted by them as casting doubt on the importance of the adjuvant regarding the slow release of antigen at the injection site (Magnusson and Kligman, 1970).

The chromium solution is not soluble in Freund's adjuvant and forms an emulsion. Thus protection and distribution of trapped antigen would not appear to be of great importance in the chromium system.

The split adjuvant technique calling for injection of Freund's complete adjuvant 24 hours prior to injection of the allergen, failed to result in chromate sensitization. Using BPS, a stronger allergen, Magnusson and Kligman (1970) injected adjuvant one, 2, 3, 4 and 6 days prior to the injection of BPS, and con-

TABLE III

MAJOR DIFFERENCES IN SENSITIZATION TECHNIQUES

	Initial injection							Reinforcement		
	Site	Route	Total volume at each site	No. of sites	Total M chromium in initial injections procedure	Adjuvant: Mixed	Adjuvant: Separate		Approximate total $K_2Cr_2O_7$	Sensitivity
Gross	Nuchal area	Subcutaneous	1.0 cc	1	0.002	×		Two injections of 0.002 M in Freund's in neck	5.1×10^{-3} M	100%
Polak	Not specified	Intramuscular	1.0 cc	5	0.003	×		Two injection of 0.09 M in saline. Also topical paintings	0.17 M	25%
Maguire adjuvant first	One flank	Intradermal	0.6 cc	5	0.005		×	Topical paintings or two intradermal injections of 0.004 M $K_2Cr_2O_7$	5×10^{-3} M	none
Antigen first	One flank	Intradermal	0.6 cc	5	0.005		×	Topical paintings or two intradermal injections of 0.0004 M $K_2Cr_2O_7$ solution	5×10^{-3} M	100%

sistently achieved lower sensitization rates than those achieved by simultaneous injection.

Considering the chromium system, it has been shown that hexavalent chromium is reduced *in vitro* before it is bound to protein. It is possible that the redox reaction with Cr_{VI}, as probably occurs *in vivo*, may be of such significance in terms of time that tissue primed 24 hours previously with Freund's complete adjuvant may no longer be capable of immunologic enhancement.

It is also possible, as recently suggested by Pass and Marcus (1970), to explain the mechanism of formaldehyde sensitivity that chromium might alter guinea pig proteins in such a way as to render them antigenic, irrespective of any complexing with them. If in delayed hypersensitivity the initial contact between immunologically competent cells and antigen takes place in the skin, as suggested by Turk (1967), the attraction of these competent cells by adjuvant should be optimally synchronized with the formation of antigen.

The striking disparity between a relative lack of success in sensitizing animals by the method of Polak *et al.*, and complete success by the antigen-first method of the split adjuvant technique as well as by the method of Gross, suggests several important differences in protocol that may explain the differences in sensitization (Table III).

In comparing intradermal, subcutaneous and intramuscular routes of sensitization with potent allergens, Magnusson and Kligman (1970) found the intradermal route the most effective, followed by the other two, respectively. The fact that the intramuscular route was employed by Polak *et al.* in their technique may be a factor in the relative lack of success in sensitization. (The volumes used in the method of Gross preclude the feasibility of intradermal injections.)

The three methods are comparable in regard to the concentrations of chromium in initial injection procedures. However, reinforcement procedures differ substantially. The Gross technique employs the adjuvant–antigen mixture on two subsequent occasions at a concentration identical to that used in the first injection. Following the Maguire technique small doses of a dilute concentration of chromium are injected intradermally, there being no further contact with adjuvant. The technique of Polak requires a rather concentrated solution, without the injection of adjuvant intradermally. Although sensitization rates generally increase when doses of antigen are increased, it is possible that significantly greater quantities of chromium in a rapidly diffusible form may potentiate desensitization rather than sensitization.

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